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Feruloyl esterase hydrolysis and recovery of ferulic acid from jojoba meal☆

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Abstract

There is growing interest in recovering ferulic acid from plant sources for use as feedstock for several high-value applications. Jojoba meal was examined as a potential source of ferulic acid. The feruloyl esterase domain of the *Clostridium thermocellum* cellulosomal xylanase was employed to hydrolyze ferulic acid from defatted jojoba meal. Esterase treatment produced 6.7 g of ferulic acid/kg of jojoba meal. The predominant source (86%) of the ferulate was found to originate from the meal's water-soluble simmondsin fraction. Seven feruloyl simmondsin species from jojoba meal were identified by liquid chromatography–mass spectroscopy. Only one species, a didemethylsimmondsin ferulate, displayed an enzymatic hydrolysis rate distinctly faster than the other feruloyl simmondsins. Complete hydrolysis of all feruloyl simmondsin species was achieved in 24–48 h at 60 °C with a 100:1 meal:enzyme weight ratio. Ferulic acid was efficiently recovered from the medium by ethyl acetate extraction. The recovered ferulic acid was readily converted to ethyl ferulate, demonstrating a facile procedure for producing a valuable product from defatted jojoba meal.
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Keywords: Simmondsin; Feruloyl esterase; Ferulic acid

1. Introduction

Seeds of the jojoba plant (*Simmondsia chinensis*) are a source of valuable waxes used in skin and hair care products. The defatted meal, while rich in protein cannot be fed to animals unless a water-soluble toxic fraction, called simmondsin, is first removed (Cokelaere et al., 1992). The simmondsin fraction has been characterized as a family of cyanocyclohexyl glycoside compounds (Elliger et al., 1973, 1974). The simmondsin

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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fraction represents 9–10% of the dry weight of the defatted meal (Holser and Abbott, 1999). Simmondsin extraction allows defatted meal to be utilized as animal feed. Finding high-value uses for the extracted simmondsin would significantly improve the economics of the meal extraction process. Medicinal uses for jojoba meal's simmondsin fraction have been suggested, but have yet to be realized (Cokelaere et al., 1992).

Ferulic acid is a phenolic compound distributed widely throughout the plant kingdom. It has potential uses as a precursor to natural vanillin and as an UV absorber and antioxidant in skin care formulations (Taniguchi et al., 1999; Compton and Laszlo, 2002; Vandamme and Soetaert, 2002; Rouhi, 2003). Many studies suggest ferulic acid may have beneficial health effects (Ou and Kwok, 2004). Typically, ferulic acid is associated with the complex polymers of the plant cell wall in relatively low quantity, thus, making its recovery from such sources economically unfavorable (Clifford, 1999). There are a few known instances where ferulic acid is found bound to small molecules in relatively high concentrations, such as the phytosterol/phytostanol fraction of rice, corn, and barley oils (Taniguchi et al., 1999). A substantial fraction (16–20%) of the jojoba simmondsin fraction is feruloylated (Van Boven et al., 1994, 1995; Holser and Abbott, 1999), suggesting this as a potential new source of ferulic acid. Thus, ferulic acid derived from the simmondsin fraction may provide additional value to the jojoba meal extraction process.

In the present study, a method to liberate enzymatically ferulic acid from jojoba meal is examined. The *Clostridium thermocellum* cellulosome is an extracellular multiprotein complex with endo- and exocellulase, xylanase, β-glucanase, and feruloyl esterase activities (Blum et al., 2000). A recombinant feruloyl esterase domain from the complex is readily expressed in *Escherichia coli* and the produced esterase is easily isolated (Blum et al., 2000). This thermostable feruloyl esterase (FAEZ) is active with a variety of substrates from soluble xylan fragments to intact cell walls, and thus, potentially reactive with simmondsin ferulates. Selective enzymatic release of ferulic acid should facilitate the recovery and isolation of this valuable product. A facile approach for recovery of ferulic acid and conversion to ethyl ferulate is also investigated.

2. Experimental

2.1. Materials

Defatted jojoba meal and crude simmondsin extract were obtained from MGP Ingredients, Inc. (Atchison, KS). The simmondsin fraction was prepared by solvent extraction (Holser and Abbott, 1999). Meal and simmondsin extract were used without further treatment. Exposure of meal and meal extracts to light was minimized, except where noted. Purified simmondsin 2′-*trans*-ferulate was a generous gift of Professor M. Van Boven (Katholic University, Leuven, Belgium). Ferulic acid, ethyl ferulate (ethyl-4-hydroxy-3-methoxy cinnamate), acetonitrile, and ethanol were purchased from Sigma–Aldrich. Hydrochloric acid (HCl) was purchased from Fisher Scientific. FAEZ was expressed in *Escherichia coli* and isolated as described previously (referred to as FAE_{XynZ} in Blum et al., 2000). The purified enzyme had an activity of 925 U/g with methyl ferulate as the substrate.

2.2. High performance liquid chromatography (HPLC)

Simmondsin ferulate species and ferulic acid were determined using a Thermo Separations Products (San Jose, CA) HPLC system consisting of a AS3000 autosampler, P4000 pump, SCM1000 solvent degasser, UV6000LP diode array detector, and a Prodigy C8 column (5 μm, 250 mm × 4.6 mm; Phenomenex, Torrance, CA). Samples were diluted into methanol and passed through Gelman 0.45 μm 13LC PVDF syringe filters prior to injection. The injection volume was 10 μl. Ferulic acid and ferulate-containing species were detected at 325 nm (7 nm bandpass). Calibration standards were prepared from pure ferulic acid, ethyl ferulate, and simmondsin 2′-*trans*-ferulate.

For determination of ferulic acid, a binary gradient system based on acetic acid, water, butanol, and methanol was used as detailed previously (Compton et al., 2000). Simmondsin ferulate species were eluted from the column with 0.25% aqueous acetic acid (solvent A) and methanol (solvent B) using a linear gradient of 70/30 A/B to 30/70 A/B over 20 min.

HPLC–mass spectroscopy was conducted with the aforementioned HPLC equipment interfaced with a Finnigan (San Jose, CA) MAT-LCQ instrument operated in the negative ion mode. The liquid feed exit-

ing the diode array detector was passed through a 1/10 flow splitter and directed into the atmospheric pressure chemical ionization interface. Ion detection parameters were tuned on directly injected simmondsin 2′-*trans*-ferulate.

2.3. Feruloyl esterase assay

FAEZ catalysis was examined at 60 °C in 50 mM sodium citrate at pH 6.0. Following predetermined intervals of incubation of meal or simmondsin extract with FAEZ, aliquots were diluted 10-fold into methanol to stop the reaction. Diluted samples were refrigerated until analyzed. The extent of hydrolysis of individual feruloyl simmondsins was determined by the decreased area of the species' peak in the HPLC chromatogram and expressed as a percentage of the area of the peak with the same retention time from a sample not treated with enzyme: hydrolyzed amount = (initial area − final area)/initial area.

2.4. Ferulic acid recovery

Crude simmondsin extract (20 g) was treated with 400 mg of FAEZ in 200 ml of water at 60 °C for 2 d. The solution initially was adjusted to pH 6.0 by addition of dilute KOH. Following the enzymatic hydrolysis step, the solution was adjusted to pH 3.0 with dilute HCl and then filtered (Whatman 52 cellulose) to remove solids. Ferulic acid was extracted from the supernatant with ethyl acetate (200 ml, twice). Ethyl acetate was removed under vacuum, which produced an amber oil. This extract was solubilized in 10 ml of ethanol. Ethanol solubilized ferulic acid was converted directly into ethyl ferulate with the addition of HCl catalyst (Section 2.6).

2.5. Nuclear magnetic resonance (NMR)

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX-400 (Karlsruhe, Germany) with a 5 mm dual proton/carbon probe (400 mHz ^{1}H/100.61 mHz ^{13}C).

2.6. Ferulic acid esterification

Reaction was performed under nitrogen using standard Schlenk line techniques. Ferulic acid (1.0 g, 5.15 mmol) was suspended in an excess of ethanol (10 ml, 171 mmol) in a sealed 50-ml Schlenk flask. Varied amounts of HCl were added and the reactions were shaken at 200 rpm and at 60 °C. The ferulic acid dissolved to form translucent, faint pale-yellow solutions. Samples (5 μl) were diluted 4000-fold into ethanol, filtered, and analyzed by HPLC to monitor reaction conversions. After completion, the reactions were neutralized with 0.5 M NaOH and reduced to dryness under vacuum at 60 °C. The syrupy residue was dissolved in 150 ml of acetonitrile, and then the solution was filtered through a silica bed to remove dark contaminates and NaCl. The silica bed was washed with two 20 ml portions of acetonitrile. The faintly yellow filtrate was dried under vacuum to produce an off white solid. Yield: 1.1 g (92% based on ferulic acid). ^{1}H NMR ($CDCl_3$) δ 7.52 (d, J = 16 Hz, 1H), 6.96 (dd, J = 2 and 8 Hz, 1H), 6.92 (d, J = 2 Hz, 1H), 6.82 (d, J = 8 Hz, 1H), 6.20 (d, J = 16 Hz, 1H), 4.16 (dd, J = 7 and 14 Hz, 2H), 3.79 (s, 3H), 1.24 (t, 3H).

3. Results and discussion

3.1. Analysis of jojoba meal extracts

Reverse-phase chromatography of aqueous defatted jojoba meal resolved several feruloyl simmondsin species (Fig. 1), some of which have not been described previously in the literature. All of these species had ultraviolet absorption spectra consistent with fer-

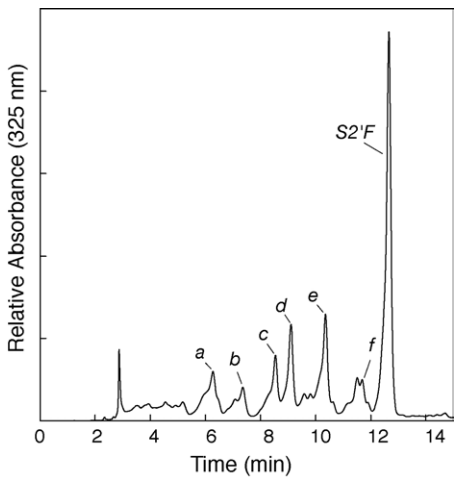

Fig. 1. Chromatogram of water-soluble fraction from defatted jojoba meal. The major simmondsin ferulate species are labeled. Refer to Table 1 for identification of the labeled species.

Table 1
Identification of feruloyl simmondsin species in chromatogram of jojoba meal aqueous extract

Label	*m*/*z*	Tentative identification
a	522 $[M-1]^-$	Didemethylsimmondsin ferulate
b	536 $[M-1]^-$; 175	Demethylsimmondsin ferulate
c	522 $[M-1]^-$	Didemethylsimmondsin ferulate
d	550 $[M-1]^-$; 175	Simmondsin ferulate
e	536 $[M-1]^-$	Demethylsimmondsin ferulate
f	550 $[M-1]^-$; 337; 175	Simmondsin ferulate
S2′F	550 $[M-1]^-$; 337; 175	Simmondsin 2′-*trans*-ferulate

Note letter designation in Fig. 1.

ulate esters (long-wavelength absorption maximum at 325 nm), and some had a pronounced feruloyl fragment (175 *m/z* in negative ion mode) present in their mass spectra (Table 1). The largest peak co-eluted with authentic simmondsin 2′-*trans*-ferulate (structure shown in Fig. 2) and its *m*/*z* value was consistent with this identification. Van Boven et al. (2000) similarly observed simmondsin 2′-*trans*-ferulate to be the principle ferulate-containing simmondsin species in jojoba meal extracts. In addition to simmondsin 2′-*trans*-ferulate, two other simmondsin ferulate isomers were identified (labeled d and f in Fig. 1) based on their *m*/*z* values. Simmondsin 3′-*trans*-ferulate is known to be present in such extracts (Van Boven et al., 1994). It

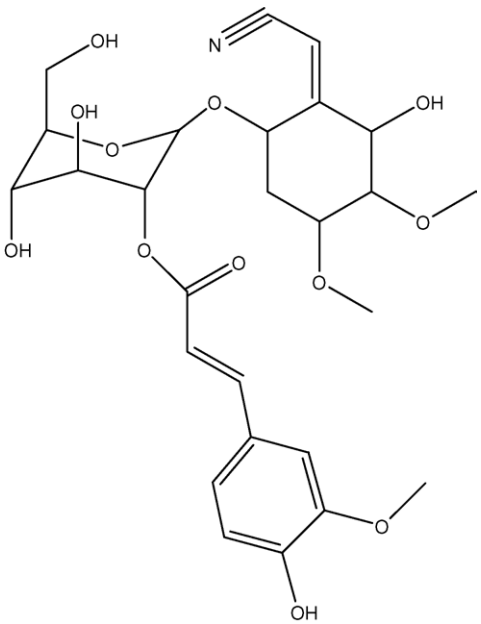

Fig. 2. Structure of simmondsin 2′-*trans*-ferulate.

may be one of the two isomers (either d or f). Simmondsin *cis*-ferulates are present in light-exposed extracts (Van Boven et al., 1996), but the present samples were not similarly treated. Deliberate light exposure of extracts (30 min with a xenon lamp) decreased the intensity of the major peaks (labeled in Fig. 1), while producing many new peaks in the chromatogram (not shown). Therefore, it is likely that none of the major species identified in Fig. 1 represents a *cis*-feruloyl simmondsin. Thus, the structure of the other simmondsin ferulate positional isomer is unknown, but perhaps may be a 4′- or 6′-feruloyl substituted simmondsin.

In addition to the various simmondsin ferulate isomers, the jojoba meal extracts contained significant quantities of feruloyl demethylsimmondsin (chromatogram peaks b and e) and didemethylsimmondsin (peaks a and c). The positional isomers 4-demethylsimmondsin 2′-*trans*-ferulate and 5-demethylsimmondsin 2′-*trans*-ferulate have been described by Van Boven et al. (1995, 1996), but the ferulate adducts of 4,5-didemethylsimmondsin apparently are newly identified here.

Peaks a, c, and e did not have detectable feruloyl fragments in their mass spectra. If the conjugating species with simmondsin was caffeic acid instead of ferulic acid, this could account for the presence of multiple occurrences of species with the same parent mass (522 or 536 *m*/*z*). However, the caffeic acid fragment (161 *m*/*z*) was not observed in any of the designated peaks in the HPLC–MS chromatogram. Furthermore, no caffeic acid was detected in the HPLC chromatogram of feruloyl esterase-treated simmondsin extract (Section 3.4). Thus, there is no evidence to support the hypothesis that any of the putative ferulate conjugates of simmondsin are caffeic conjugates.

A small amount of ferulic acid also was present in jojoba meal extracts. It eluted just prior to, and incompletely resolved from peak f in the HPLC chromatogram shown in Fig. 1. For the purposes of quantification of ferulic acid, an alternative elution method was employed (Compton et al., 2000) to provide baseline resolution of ferulic acid from the feruloyl simmondsins (not shown).

3.2. Feruloyl esterase treatment of jojoba meal

Ferulic acid is slowly released over several days from defatted jojoba meal suspended in buffer, sug-

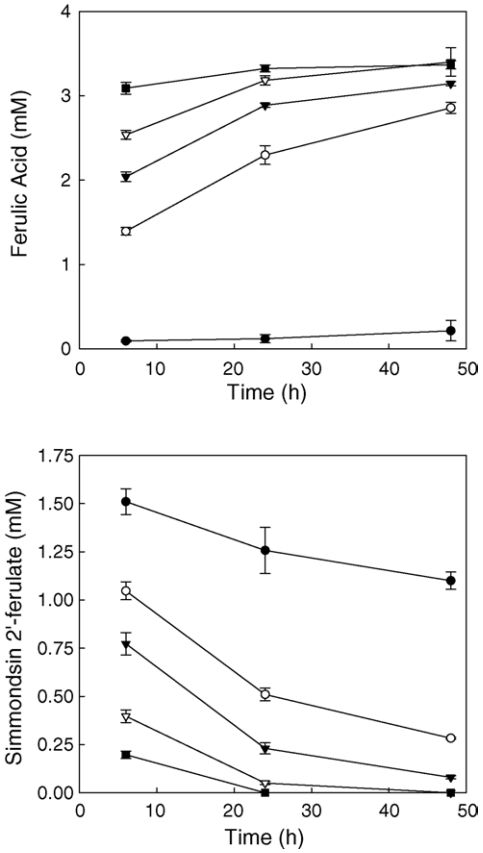

Fig. 3. Changes in ferulic acid (upper panel) and simmondsin 2′-*trans*-ferulate (lower panel) concentrations during treatment of defatted jojoba meal (100 mg/ml) with FAEZ. FAEZ concentrations (mg/ml): (●) 0.0, (◯) 0.25, (▼) 0.5, (▽) 1.0, and (■) 2.0.

gesting the presence of a small amount of intrinsic feruloyl esterase activity in the meal (Fig. 3, upper panel). Adding FAEZ to the suspension greatly increased the amount of ferulic acid produced (Fig. 3, upper panel). FAEZ (2.0 mg/ml) treatment released 6.7 g of ferulic acid/kg of meal in 24 h, beyond which little or no further ferulic acid was released. Lower concentrations of the enzyme released ferulic acid at slower rates. The disappearance of simmondsin 2′-*trans*-ferulate from the suspension with FAEZ treatment mirrored production of ferulic acid (Fig. 3, lower panel). Simmondsin 2′-*trans*-ferulate accounts for 44% of the ferulic acid released from the meal. No feruloyl simmondsins remained in the meal suspension after 48 h of 2.0 mg FAEZ/ml treatment (data not shown), indicating that all species were susceptible to enzymatic hydrolysis.

The susceptibility of feruloyl simmondsin to enzymatic hydrolysis suggests that much of the ferulic acid released from jojoba meal by FAEZ arises from the simmondsin fraction. To test whether the simmondsin fraction was the sole source of ferulic acid, the meal was extensively washed with water to remove all traces of feruloyl simmondsins (confirmed by HPLC). This simmondsin-depleted meal was then subjected to FAEZ treatment (100 mg meal/ml, with 2.0 mg FAEZ/ml in citrate buffer for 48 h). The concentration of ferulic acid produced from the simmondsin-depleted meal was only 14% of the amount generated from the complete (defatted) meal. This finding indicates that a small fraction of the ferulic acid produced by FAEZ treatment comes from an insoluble fraction of the meal (i.e., cell wall polysaccharides and other cell wall components), while the majority of the ferulic acid (86%) derives from the simmondsin fraction. Furthermore, the rate of ferulic acid release from the insoluble meal fraction was similar to that observed with the whole meal (data not shown), indicating that there is no great preference of the enzyme for one or the other type of substrate (soluble feruloyl simmondsins or the insoluble feruloyl cell wall matrix).

Complete alkaline hydrolysis of the jojoba meal with 1.0 M KOH (60 °C for 24 h, under nitrogen) released 9.1 g of ferulic acid/kg of meal. Therefore, FAEZ treatment fails to release approximately 26% of the ferulic acid content of the meal. This indicates that FAEZ can completely hydrolyze soluble feruloyl simmondsins, but not all cell wall-bound ferulates. Note that alkaline hydrolysis of the meal also releases amounts of coumaric acid comparable with that of ferulic acid, whereas FAEZ treatment releases only trace amounts of coumaric acid. The selectivity of the enzymatic process allows a more facile ferulic acid recovery process (Section 3.5) than would be had with alkaline hydrolysis.

3.3. Initial hydrolysis kinetics of feruloyl simmondsins

A crude simmondsin extract was used to examine whether FAEZ demonstrates selectivity towards any of the feruloyl simmondsins. The crude extract was treated briefly (20 min) with enzyme and then examined by HPLC to determine the extent of hydrolysis of individual species. Only a modest variation in ini-

Table 2
Extent of hydrolysis of feruloyl simmondsin species after treatment with FAEZ for 20 min[a]

Label[b]	Hydrolyzed (%)
a (DDSF)	12.1 ± 1.7[c]
b (DSF)	5.7 ± 1.1
c (DDSF)	23.7 ± 4.2
d (SF)	6.1 ± 3.4
e (DSF)	7.1 ± 1.6
S2′F	9.4 ± 1.6

[a] Conditions: crude simmondsin extract, 40 mg/ml; 1.0 mg FAEZ/ml.
[b] Refer to Fig. 1 and Table 1.
[c] Standard deviation ($n = 3$).

tial hydrolysis rates among the various species was observed. One of the didemethylsimmondsin ferulate isomers (peak c) turned over two- to three-fold faster than the other species (Table 2). Hydrolysis of the simmondsin ferulate peak f could not be followed as it was obscured by the ferulic acid generated by the FAEZ treatment.

The FAEZ substrate-binding site has been examined in detail (Schubot et al., 2001). The hydrophobic ferulic acid-binding pocket, which is open and solvent exposed, aligns the substrate with the "catalytic triad" of the active site. An adjacent site interacts with the arabinofuranosyl moiety of enzyme's native substrate. The weak interaction between the protein and carbohydrate-binding site may account for the accommodation of the glucosyl portion of feruloyl simmondsins in the enzyme's reaction site. The limited selectivity shown by FAEZ with feruloyl simmondsins is consistent with this interpretation. Although ferulic acid glucosyl esters are found in other natural plant substances (Masuda et al., 2000), we are unaware of any reported feruloyl esterases displaying activity with these substrates.

3.4. Complete ferulate hydrolysis of simmondsin extract

Extended FAEZ treatment of crude simmondsin extract (40 mg extract/ml, with 1.0 mg FAEZ/ml in citrate buffer for 48 h) produced a solution containing 3.4 mM ferulic acid with no residual feruloyl simmondsins. Thus, the crude simmondsin extract represents a somewhat enriched source of ferulic acid (1.7%, w/w) compared with the defatted meal (0.67%, w/w). It is noteworthy that the ferulic acid content of this crude extract is comparable with that of corn bran (3%, w/w; Saulnier et al., 1995), making it a rich natural source of enzymatically accessible ferulic acid. However, the high levels of enzyme required to release ferulate from the simmondsin fraction implies that substantial improvements to the enzyme's specific activity must be made before the process could be considered economically feasible.

3.5. Recovery of ferulic acid

Various approaches have been applied in the past to recover ferulic acid from enzymatic hydrolysate liquors. Couteau and Mathaly (1998) attempted to use activated carbon adsorption with limited success (ferulic acid purity ∼50%). We attempted to use anion-exchange resins (Dowex MSA-2 macroporous resin and Whatman QA-52 quaternized cellulose) to recover ferulic acid from FAEZ hydrolyzates of crude simmondsin extracts (performed without citrate in the reaction mixture). The effective capacity of the exchangers for ferulic acid was ∼25% of the total exchange capacity. Elution of ferulic acid with ethanolic HCl required at least three bed volumes. The resins appeared to retain a great deal of dark material after the ethanolic elution step. This approach was abandoned because of the low effective exchanger capacity, large elution volumes required to recover ferulic acid, and the potential for resin fouling by other components of the crude simmondsin hydrolysate.

A more successful approach was to solvent-extract ferulic acid from the crude simmondsin hydrolysate with ethyl acetate. Solvent extraction quantitatively separated the ferulic acid from the hydrolysate aqueous phase. The recovered ferulic acid had a chromatographic purity of approximately 80%. Final purification was most readily achieved after converting the ferulic acid to ethyl ferulate (Section 3.6).

3.6. Facile conversion of ferulic acid to ethyl esters

While the principal objective of this study was to demonstrate the validity of enzymatically liberating and isolating ferulic acid from jojoba meal, the conversion of ferulic acid to its ethyl ester is of particular interest to our group. Ferulic acid was reacted with excess ethanol using HCl as a catalyst to form ethyl ferulate.

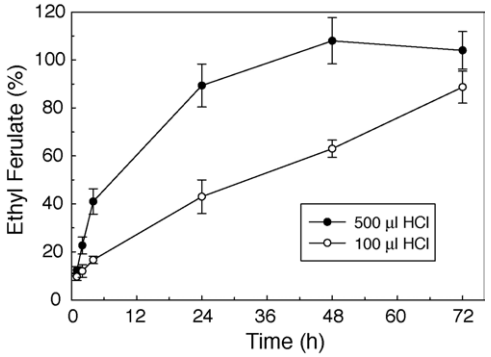

Fig. 4. Esterification of ferulic acid (1.0 g, 5.15 mmol) with ethanol (10 ml, 171 mmol) catalyzed by 500 or 100 μl of HCl (6.0 and 1.2 mmol, respectively) at 60 °C. Ethyl ferulate was quantified by HPLC.

An approximate equimolar ratio of HCl to ferulic acid resulted in a 100% conversion to ethyl ferulate after 48 h (Fig. 4). A reaction conducted with a lower HCl to ferulic acid molar ratio (1:5) resulted in slower kinetics and did not reach completion after 72 h. The ethyl ferulate was recovered with 92% yield by removal of the excess ethanol, which could be recycled. Ferulic acid recovered from FAEZ hydrolyzed simmondsin (Section 3.5) was also quantitatively converted to ethyl ferulate in ethanol using the HCl catalyst.

4. Conclusions

Jojoba simmondsin ferulates consist of at least seven different isomers, varying in the extent of methoxylation of the cyclohexyl ring and position of the feruloyl group on the glucosyl moiety. The recombinant feruloyl esterase domain from the *Clostridium thermocellum* cellulosome (FAEZ) completely hydrolyzes all seven isomers. There is additional ferulic acid in jojoba meal not associated with simmondsin that is only partially accessible to FAEZ. Of the 9.1 g of ferulic acid/kg of defatted meal, 6.7 g (74%) are released by FAEZ. The feruloyl simmondsin fraction contributes 5.8 g (86%) of the ferulic acid released from the meal. Feruloyl esterase treatment of a partially purified simmondsin fraction releases ferulic acid as the only phenolic product. The simple phenolic composition of the simmondsin fraction facilitates ferulic acid recovery, purification, and conversion into commercially valuable products. Engineering of FAEZ to improve its activity with feruloyl simmondsin substrates would greatly increase the economic feasibility of upgrading the value of jojoba meal through recovery of its ferulic acid content.

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